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# Severity of Nonbullous *Staphylococcus aureus* Impetigo in Children Is Associated with Strains Harboring Genetic Markers for Exfoliative Toxin B, Panton-Valentine Leukocidin, and the Multidrug Resistance Plasmid pSK41

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**Nonbullous impetigo is a common skin infection in children and is frequently caused by *Staphylococcus aureus*. Staphylococcal toxins and especially exfoliative toxin A are known mediators of bullous impetigo in children. It is not known whether this is also true for nonbullous impetigo. We set out to analyze clonality among clinical isolates of *S. aureus* from children with nonbullous impetigo living in a restricted geographical area in The Netherlands. We investigated whether staphylococcal nasal carriage and the nature of the staphylococcal strains were associated with the severity and course of impetigo. Bacterial isolates were obtained from the noses and wounds of children suffering from impetigo. Strains were genetically characterized by pulsed-field gel electrophoresis-mediated typing and binary typing, which was also used to assess toxin gene content. In addition, a detailed clinical questionnaire was filled in by each of the participating patients. Staphylococcal nasal carriage seems to predispose the patients to the development of impetigo, and 34% of infections diagnosed in the Rotterdam area are caused by one clonal type of *S. aureus*. The *S. aureus* strains harbor the exfoliative toxin B (ETB) gene as a specific virulence factor. In particular, the numbers ($P = 0.002$) and sizes ($P < 0.001$) of the lesions were increased in patients infected with an ETB-positive strain. Additional predictors of disease severity and development could be identified. The presence of a staphylococcal plasmid encoding multiple antibiotic resistance traits, as detected by binary typing, was associated with a reduction in the cure rate. Our results recognize that a combination of staphylococcal virulence and resistance genes rather than a single gene determines the development and course of nonbullous impetigo. The identification of these microbial genetic markers, which are predictive of the severity and the course of the disease, will facilitate guided individualized antimicrobial therapy in the future.**

Over the past few years the prevalence of streptococci as a leading cause of impetigo has dropped, whereas *Staphylococcus aureus* has become the most common causative microbial pathogen of this prevalent childhood disease (7, 8, 23). *S. aureus* is usually encountered as a nasal commensal organism, but the bacterium may ultimately induce a broad variety of skin infections. This suggests that staphylococcal carriage predisposes an individual to the development of impetigo (14, 25). Fortunately, the vast majority of these *S. aureus* strains are still broadly susceptible to antibiotics, such as fusidic acid and mupirocin (15, 19, 26).

*S. aureus* toxins are considered the main effector molecules in a wide variety of childhood exanthems. The clinical presentation is often due to the effects of various cytokines induced as a consequence of the infection process, and a variety of disease mechanisms have been postulated (20, 22). Recently, it was discovered that staphylococcal exfoliative toxin A (ETA) hydrolyzes human desmoglein-1 (1). The destruction of this important structural skin protein leads to the development of blisters below the stratum corneum, possibly facilitating bacterial proliferation beneath the physical skin barrier. Novel therapies may be based on these important findings. Consequently, there is a need for versatile laboratory systems enabling the direct or indirect detection of toxins and other virulence factors of clinical isolates of *S. aureus* (16). However, one must realize that the mere presence of a certain gene does not prove its causality in disease. Establishment of the latter requires detailed clinical studies in addition to molecular diagnostics.

We recently performed a double-blind, randomized, placebo-controlled trial into the clinical effectiveness of fusidic acid cream in the treatment of impetigo diagnosed among children in general practice (15). It showed that fusidic acid cream treatment significantly accelerated to clinical and bacteriological cure of the infection and that the main cause of impetigo was *S. aureus*. In the present study we have set out to analyze the gene and genomic variabilities among the clinical isolates of *S. aureus* found at the time of enrollment in the trial (before treatment), using pulsed-field gel electrophoresis (PFGE)-me-

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diated typing and a novel binary typing (BT) protocol. We assessed the strains for the presence or absence of toxin-encoding genes and investigated whether the virulence gene potential of the staphylococcal strains involved and staphylococcal nasal carriage per se were associated with the severity and course of the impetigo.

## MATERIALS AND METHODS

**Bacterial isolates and clinical data collection.** Koning et al. (15) described the isolation of *S. aureus* strains from children affected by impetigo in the Rotterdam region of The Netherlands. Swabs for the detection of bacteria were obtained from the nose, which is the primary ecological niche for *S. aureus*, and from the impetigo wound. A trained research nurse obtained all samples at the home visit for inclusion in the study. Ninety-eight *S. aureus* strains were analyzed by PFGE and extended BT (see below). The following clinical parameters were recorded for all patients ($n = 98$): number of lesions, location of the lesion, surface area of the lesion, temperature, lymphadenopathy (a patient was defined as positive once a palpable enlargement of the regional lymph nodes concerned was obvious), encrustation of the wound, presence of pustules, duration of infection, and swimming habits. In addition, we recorded the age, sex, ethnicity, quality of living environment, and kindergarten attendance. We also investigated the relationship between virulence gene markers of the *S. aureus* strains and the course of impetigo, focusing on clinical and bacterial cure 1 week after inclusion in the study and the start of therapy. Clinical cure was defined as the complete absence of lesions or the presence of lesions that had become dry without crusts. Bacterial cure was defined as the absence of *S. aureus* strains at the lesion. The medical ethical committee of the Erasmus Medical Center approved the trial protocol, and informed consent was obtained from the patients and their parents. In case the patients withdrew from the study protocol, evaluation visits were still carried out as scheduled.

**PFGE analysis.** PFGE analysis for *S. aureus* was performed by established protocols (27). Electrophoresis was performed in contour-clamped homogeneous electric field mappers (Bio-Rad, Veenendaal, The Netherlands) by using a 20-h program consisting of two switching blocks (10 h for 5 to 15 s and 10 h for 15 to 45 s). The buffer system consisted of 1× TBE (Tris-borate-EDTA), whereas the temperature was kept constant at 14°C. DNA macrorestriction fragment-containing blocks were prepared by embedding *S. aureus* cells and treating these in situ with lysostaphin (Sigma, Steinheim, Germany), proteinase K (Sigma), and *Sma*I (Boehringer, Mannheim, Germany). For size determination, reference samples containing phage λ concatemeric DNA (Bio-Rad) were included in all gels. After PFGE, the gels were stained with ethidium bromide and photographed. Interpretation of the banding patterns was performed visually, and types were assigned as suggested by Tenover et al. (24). In short, banding patterns differing at less than three band positions were assigned the same overall type; subtypes were identified by numbering.

**Extended BT.** BT of the *S. aureus* strains was performed with the system developed by Van Leeuwen et al. (28). Twelve strain-selective probes were immobilized on nylon strips, and reverse hybridization with chemically labeled staphylococcal DNA was performed. Signals were recorded after staining; and a 1 or 0 binary score was deduced for each of the probes, resulting in a 12-digit 1/0 genotype for each of the strains. This classical approach uses 12 probes for BT (BIN 1 to 9, 11, 14, and 15). The sequences of these probes are homologous to unknown regions in the staphylococcal genome (BIN 7), a hypothetical but not yet identified gene (BIN 2, 5, 8, and 15), the Panton-Valentine leukocidin gene (BIN 1), a repetitive motif (BIN 3), a pSK41-like multiresistance-encoding plasmid (BIN 4), the ϕSLT bacteriophage (BIN 6), the intergenic region between a tRNA synthetase and an amino acid permease (BIN 9), the 3-hydrocyacyl coenzyme A dehydrogenase gene (BIN 11), and the acetyl coenzyme A synthetase gene (BIN 14). In addition, 11 toxin gene-specific probes were included (18). These included probes for enterotoxins A, B, C, D, and E; ETA and ETB; toxic shock syndrome toxin; and hemolysins A, B, and C. All probes were generated by PCR. The validity of the results obtained by this approach was verified by including *S. aureus* strains with a phenotypically determined toxin production pattern as positive controls (results not shown). The latter strains were obtained from Max Heck and Wim Wannet (Department of Infectious Disease Epidemiology, State Institute of Public Health and the Environment, Bilthoven, The Netherlands).

**Statistical analyses.** For pairwise comparisons, Fisher's exact tests were performed by using 2-by-2 contingency tables. Two-sided *P* values were considered significant once they were $<0.05$. Numerical analyses were performed by analysis of variance. The relationship between gene markers and the course of disease was tested by both univariate analysis and stepwise logistic regression analysis. In the multivariate analysis, baseline characteristics and treatment modality (fusidic acid cream or placebo) were included in the analysis.

## RESULTS

**Genotyping of the bacterial isolates.** Ninety-eight *S. aureus* isolates from the wounds of as many patients, obtained at their inclusion visit, were genetically characterized by PFGE and BT. A novel genotype was defined on the basis of the presence of three or more band differences in the PFGE pattern, which was always accompanied by at least two differences in the BT outcome. Overall, 67 different genotypes could be identified among these strains. Thirty-nine strains were identified as belonging to a single clone, identified by similarities in the PFGE banding patterns (24). This clone was given the code 8. BT confirmed the existence of the clonal type as defined by PFGE, and BT probes BIN 1 to BIN 4 identified most of the genetic heterogeneity between PFGE type 8 and non-PFGE type 8 (diverse) strains. For all of the binary codes for the clone 8 group (except for those obtained with BIN 2, 3, and 7), exceptions to the consensus code were found. This corroborates the genetic microheterogeneity already detected by PFGE and confirms that the sequences of the BIN probes originate from intrinsically unstable regions in the *S. aureus* genome. We were unable to document significant geographic clustering of clones within the Rotterdam area (results not shown).

**Toxin gene detection.** The hemolysin A to C genes were present in essentially all of the *S. aureus* strains isolated during the Rotterdam impetigo study. The enterotoxin A, D, and E genes, in contrast, were rare. Furthermore, not all of the clone 8 strains were identical with respect to their toxin gene potentials. When the clonal isolates were compared to the other (genetically diverse) strains, the presence of ETB and enterotoxin C and the absence of the toxic shock syndrome toxin appeared to be specific for the clonal isolates.

**Nasal carriage and clinical characteristics.** Among the patients for whom nasal swab specimens were available for culture, 79% showed nasal colonization by *S. aureus* during the inclusion visit. When strain identity was assessed pairwise for each of the patients at the time of presentation, 75% had the same strain in the nose and the wound. Table 1 summarizes the data obtained by statistical comparison of bacterial and clinical characteristics. The presence of a given bacterial characteristic was not related to sex, ethnicity, occurrence of lesions on the head or the limbs, sensations of pain, frequency of impetigo, preexisting eczema, or records of boils in the family (data not shown). Table 1 highlights the characteristics that are statistically significant when the clinical signs and the presence or absence of a given gene probed by BT are compared. All 111 strains derived from wounds for which both BT and toxin detection results were available were included in the survey, irrespective of their genotype (clone 8-related and genetically heterogeneous strains). In order to circumvent clone 8-induced bias, the same statistical exercise was repeated for the strains harboring non-clone 8 genotypes. The boldface values in Table 1 highlight those parameters that retained significance upon reanalysis. These will be discussed in more detail below.

A major marker for disease severity was the presence of the bacterial ETB gene (Table 1). In particular, the number of

lesions observed and the total surface size of the lesions were highly significantly associated with the presence of ETB. Also, positivity with BT probes BIN 1 (homologous to the Panton-Valentine leukocidin gene) and BIN 4 (pSK41-like multiresistance-encoding plasmid) seemed to be a predictor for the severity of infection. Apparently, a strain harboring ETB and sequences specific for the BIN 1 and BIN 4 probes showed strong disease-inducing potential, probably due to the combined presence of virulence and antibiotic resistance genes. For ETB and BIN 1 this finding was corroborated by the absence of lymphadenopathy. The association between carriage of the ETB-positive staphylococci and kindergarten attendance disappeared upon elimination of clonal type 8 strains from the analysis. Some additional conclusions can be drawn from Table 1. The presence of the genes encoding enterotoxins C and D predisposed individuals to pustule formation. Strains harboring these genes did not seem to be spreading through kindergarten-associated infections. Furthermore, different age groups were colonized with different types of *S. aureus* isolates. Finally, the sequence specific for the BIN 8 probe was associated with wound encrustation. Although the BIN 8 nucleotide sequence corresponds to that of a gene in the *S. aureus* chromosome to which a function has not yet been attributed and that is ill defined at present, the observed association with impetigo implies that additional research into the precise nature of this potential virulence gene is warranted. BIN 2, whose sequence matches that of another hypothetical gene, seems to be associated with pustule formation. Again, further studies into the nature of this gene are urgently required. Multivariate analysis showed that the presence of the gene for ETB was the clinically most relevant predictor of disease severity.

**Relation between gene markers and clinical cure.** None of the univariate relationships between gene markers and clinical cure after 1 week reached statistical significance, nor did nasal staphylococcal carriage. In stepwise multivariate logistic regression analysis, apart from treatment modality, only the presence of the BIN 4-specific sequence contributed significantly to clinical cure ($\beta = -1.68$; $P = 0.005$), with a lower cure rate for patients that were infected with BIN 4-specific sequence-positive strains. For bacterial cure after 1 week (data were available for 88 patients), the presence of ETB gene- and BIN 8-specific sequence-positive strains turned out to be statistically related. When the ETB gene was present, 40% of the patients were cured after 1 week, whereas in the absence of the gene, 60% were cured after 1 week ($\chi^2 = 5.25$; degrees of freedom = 1; $P = 0.03$). When strains were positive for the BIN 8-specific sequence (which was the case for 84 of 88 children), 60% were cured within a week. Among the four patients with a BIN 8-specific sequence-negative strain, none were cured ($\chi^2 = 5.51$; degrees of freedom = 1; $P = 0.03$). In the regression analysis, apart from treatment modality, only the BIN 7-specific sequence contributed to some extent, but this was not statistically significant.

## DISCUSSION

Nasal carriage of *S. aureus* is an important predisposing factor for the development of staphylococcal infections (14). The 79% carriage rate among our impetigo patients exceeds the average carriage rates found in many other studies, sug-

TABLE 1. Association between virulence gene markers of *S. aureus* strains cultured at inclusion of patients in a randomized placebo controlled study on the efficacy of fusidic acid and clinical and demographic features of children with impetigo[a]

| Characteristic | SEC | SED | SEE | ETA | ETB | TSST | BIN 1 (PVL) | BIN 2 (hyp. gene) | BIN 4 (pSK41) | BIN 8 (hyp. gene) |
|---|---|---|---|---|---|---|---|---|---|---|
| Age (yr) | 2.8–5.5 (**<0.001**) | | | | 6.8–4.2 (<0.001) | | 6.1–4.3 (0.001) | | 5.9–4.2 (0.002) | |
| Duration (days) at first visit | | | | | | 13.8–8.7 (**0.02**) | | | | |
| No. of lesions | | | | | 17.7–9.9 (**0.002**) | | 15.4–10.1 (0.03) | | 15.9–8.8 (**0.003**) | |
| Surface area of lesion ($cm^2$) | | | | | 11.6–3.9 (**<0.001**) | | 9.3–4.2 (**0.001**) | | 8.7–4.0 (**0.003**) | |
| Lymphadenopathy (% of patients) | | 100–39 (**0.03**) | | | 20–53% (**0.003**) | 73–37 (0.03) | 30–51 (0.04) | | | |
| Temp (°C) | | | | 36.1–36.6 (**0.01**) | | | | | | |
| % Patients with: | | | | | | | | | | |
| Encrustation | | | | | | | | | | 95–80 (0.03) |
| Pustules | 41–16 (0.02) | 75–18 (0.02) | | | | | 12–26 (**0.05**) | 50–17 (0.03) | | |
| Lesions on trunk | | | | | 51–24 (**0.005**) | | | | | 31–80 (0.04) |
| Family members or peers infected | | | | | 35–12 (**0.005**) | | | | | |
| Home in an old house | | | 100–34 (0.03) | | | | | | | |
| Kindergarten attendance | | | | | 5–27 (0.005) | | 6–31 (0.001) | | | |
| Kindergarten attendance and age >4 yr | 53–80 (0.02) | 25–78 (0.04) | | | 87–70 (0.05) | | | | 85–66 (0.02) | |
| Swimming participation | | | | | 68–46 (0.03) | | | 27–57 (0.03) | 62–43 (0.04) | |

[a] Data are for 98 combinations of completely typed *S. aureus* strains together with a full complement of patient information. The pairs of figures listed indicate the average value of the parameters investigated for the patients harboring strains including the markers versus those that did not. In the case of the number of lesions, for instance, the average number of lesions was 17.7 for patients infected with ETB-positive strains, whereas for patients infected with an ETB-negative strains the number of lesions averaged 9.9. Only statistically significant figures are included. No association with any of the listed disease parameters was found for the genes encoding enterotoxins A and B and hemolysins A, B, and C and for and BIN probes 2, 3, 5 to 7, 11, 14, and 15. Pairs of figures indicate the probe-positive (left) versus the probe-negative (right) results. *P* values are given in parentheses. For boldface *P* values, significance was retained upon exclusion of the clone 8 PFGE types of *S. aureus*. PVL, Panton-Valentine leukocidin; pSK41, multiresistance-encoding plasmid pSK41; hyp. gene, hypothetical gene segment; SEC, SED, and SEE, enterotoxins C, D, and E, respectively; TSST, toxic shock syndrome toxin.

gesting that *S. aureus* carriage predisposes individuals to the development of impetigo. However, the ultimate proof for this should be based upon prospective studies, since our study design does not enable a distinction between cause and effect. On the basis of the present data set, it is not possible to definitely conclude that nasal carriage precedes skin infection. This would have required sequential bacterial cultivation prior to impetigo development. This is an impracticable approach.

The toxin gene repertoire of a given *S. aureus* strain has been suggested to be an important denominator for the strain's capacity to induce skin disease (3, 6, 17, 21). In particular, the antigenically distinct subtypes of staphylococcal ETA and ETB are putatively involved in the development of bullous impetigo. Previous studies in Japan have demonstrated that approximately 70% of all clinical isolates produced either of these toxins, whereas among strains isolated from patients with atopic dermatitis and furuncles, these rates were 3 and 0%, respectively (13). In Japan, ETA was present in 57 of 144 (40%) strains tested, ETB was present in 36 of 144 (25%) strains, and both genes were present in 7 of 144 (5%) strains. Our values were 12 of 98 (12%), 36 of 98 (37%), and 6 of 98 (6%) strains tested, respectively. This may suggest geographical differences in gene incidence, but it also suggests that both ETA and ETB are important factors in the development and progression of impetigo. The fact that ETB may be plasmid borne probably contributes to its ease of dissemination (29). Also, an association between exfoliative toxin gene content and impetigo was documented in Italy (4, 5). It must be emphasized that the values mentioned above highlight the fact that not all *S. aureus* strains causing impetigo harbor, for instance, the ETB gene. This implies that additional factors, either host or pathogen derived or both host and pathogen derived, affect the development of impetigo. This suggests that impetigo is a multifactorial syndrome, for which we provide evidence from at least the bacteriological point of view.

We have shown here that, analogous to the already documented clinical importance of ETA in bullous impetigo, the structurally similar toxin ETB contributes significantly to the development of nonbullous impetigo. In particular, the surface area and the number of impetigo lesions were increased in patients with ETB-positive *S. aureus* infections. The epidemiological association between the presence of the bacterial gene and disease severity was very clear. Our study shows that clonal dissemination of certain bacterial genotypes is important in this respect, explaining a large proportion of disease cases. Additional clinical-microbiological markers were identified by BT. The sequence of one of these, BIN 4, displays homology to the sequences of the VRS plasmids belonging to the pSK41 family (2, 9). How these multiresistance-encoding conjugative plasmids, which harbor all requirements for gene transfer and various insertion elements, relate to the severity of skin disease is unclear at present. However, its apparent relatedness to a delay of cure is probably due to the antibiotic resistance that is associated with this class of plasmids. For another important marker that we discovered, the sequence specific for BIN 1, the relation to disease severity is more obvious: this binary probe shares sequence homology with a Panton-Valentine leukocidin located on prophage ϕPV8 (12). The possible virulence effect of such a molecule is obvious, as was also demonstrated in two recent studies showing an increased incidence of the leukocidin gene among *S. aureus* strains isolated from impetigo patients (10) and general clinical isolates (11). Moreover, Lina et al. (17) demonstrated that this leukocidin appears to be specifically associated with necrotic lesions of the skin and mucous membranes.

In conclusion, we have shown here that impetigo among patients living in a geographic region of limited size can be caused by both clonally related and unrelated *S. aureus* strains. For the first time, a relation between the virulence gene content and the severity of impetigo has been demonstrated. Most particularly, we have demonstrated here that various virulence factors are involved in impetigo and that the severity of disease is probably dependent upon the combined presence and activities of these various factors. Independent of PFGE-defined staphylococcal clonality, bacterial toxin and virulence gene contents are important factors related to the severity and sometimes even the course of the disease. Further improvement of microbial gene typing strategies, such as those used in the present study, may result in prospective therapeutic interventions guided by molecular assessment of the virulence of the infectious agent.

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